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13. Abstract *(Maximum 200 Words)* (abstract should contain no proprietary or confidential information)
The major goal of the research was to develop a novel methodology for improving T cell epitopes. The underlying hypothesis is that T cells capable of recognizing tumor-associated antigens (TAA) are present but often difficult to activate. However, once activated such T cells might be effective against tumors due to the less stringent triggering requirements of mature effectors. We developed a novel bacterial expression system for screening the epitopes of PSA, a known TAA. We have employed a saturation mutagenesis technique to a PSA peptide epitope identified. Expression libraries constructed corresponding to each position in the peptide were screened functionally. Plasmids from clones showing enhanced activity were sequenced. Peptides were synthesized and tested in a functional assay and showed enhanced activity with the T cell hybridoma. Using a competitive binding assay for MHC binding, we showed that some mutants enhance T cell activation by binding better to MHC whereas others appear to improve interaction with the T cell receptor. Immunization with a mixture of altered peptide ligand and the wild-type peptide can enhance the frequency of responding cells. The findings show that altered peptide ligands can be discovered using this novel methodology and should be applicable to other tumor antigens.

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INTRODUCTION

The development of effective immunotherapy for cancer has long been a goal of tumor immunologists. Cytotoxic T cells (CTL) may effectively kill tumor cells expressing tumor associated antigens (TAA) if the T cells are initially activated sufficiently since effector T cells have much less stringent activation requirements than naïve T cells. However, T cells are often poorly activated to these tumor-associated antigens. One approach to improving the activation of CTL is to modify the target epitopes so that the T cells are activated more effectively, yet retain activity against the original TAA. A significant limitation of this strategy is that it is difficult to test all the possible epitopes in an unbiased fashion. The major goal of the proposal is to develop a novel methodology to improve T cell epitopes of PSA that could be used ultimately in the immunotherapy of prostate cancer and should be applicable to many other cancers.

BODY

Approved Tasks

The following tasks were outlined in the approved statement of work for this grant:

- Task 1. Generation of CTL and class I restricted hybridomas (1-18 months)
- Task 2. Identification of class I restricted epitopes (months 1 - 24)
- Task 3. Develop and screen library of mutant epitopes (months 6-30)
- Task 4. Testing and analysis of improved epitopes (months 24-36)

Research Accomplishments associated with the above tasks

Task 1. Generation of CTL and class I restricted hybridomas

We have successfully generated CTL lines and hybridomas for a class I restricted epitope of PSA. The hybridoma PSA-HI has been characterized and has been used to accomplish the subsequent tasks as outlined below. This task has been successfully completed. This work is described in more detail in the appended reprint (1).

Task 2: Identification of class I restricted epitopes

The hybridoma PSA-HI has been employed in the proposed solid phase antigen presentation system assay in which bacterially expressed proteins are used as the source of the antigen. Using this approach, we have successfully identified a class I restricted epitope of PSA, HPQKVTKFML, abbreviated HL10. The identification of the epitope has been unequivocally demonstrated by the synthesis of the peptide. Moreover, we have shown that this epitope can be recognized by T cells infiltrating tumors, demonstrating that this epitope is important *in vivo* responses. For a more detailed description of these results, a paper that has been published which incorporates these results is appended to this report (1).

Task 3: Develop and screen library of mutant epitopes

We hypothesized that altered peptides with enhanced activity might either improve MHC binding or improve T cell recognition. In order to test directly whether the hybridoma system employed in the screen proposed could detect and respond appropriately to either type of change we utilized SIINFEKL, a well characterized H2-K^b-restricted epitope, which is derived from ovalbumin. Previous work (2) demonstrated that T cell lines specific for SIINFEKL yield

different degrees of lysis on specific mutant peptides derived from SIINFEKL (SL8). One of the mutants contained an alanine for a phenylalanine substitution at position 5 ($SL8_{5A}$), which impairs the ability of the peptide to bind to its MHC molecule, while the other mutant contained an alanine for a lysine substitution at position 7 ($SL8_{7A}$) which interferes with the ability of the peptide to interact with its T cell receptor. As previously reported, to determine how well the β-galactosidase reporter T cell hybrids parallel CTL cell lines in responding to mutant peptides, we utilized B3Z, a class I restricted hybrid specific for SIINFEKL, along with the mutants described above (Figure 1).

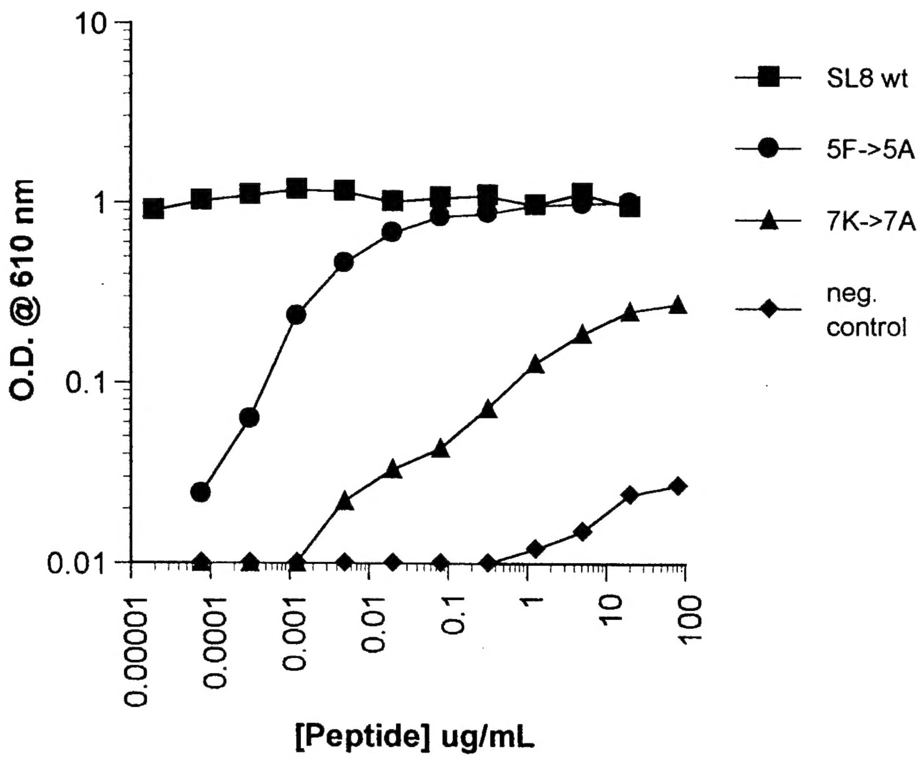

Figure 1: Activity of hybrids with mutants derived from the wild type peptide.
Activation of a class I restricted T cell hybridoma, B3Z, was assessed using mutant peptides derived from the wild type peptide of B3Z, SIINFEKL (SL8). $SL8_{5A}$ is a MHC binding impaired mutant, while $SL8_{7A}$ is impaired in its ability to interact with SL8 specific T cell receptors. Peptides at decreasing concentrations were added to 1 x 10^5 B3Z in the presence of 1 x 10^5 Ana-1, an $H2K^b$ expressing macrophage cell line. After 12 hours at 37°C, β-galactosidase production was detected using CPRG, a substrate for β-galactosidase.

As can be seen in Figure 1, the wild type peptide SL8 stimulates very effectively compared to the negative control. In contrast, the altered peptide ligands (APLs) of SL8 are much less stimulatory. Thus, the β-galactosidase containing hybrids utilized in this screen are sufficiently robust and quantitative to discriminate between peptides with known differences in their ability to stimulate T cells. Note in this case that the APL is less stimulatory than the wild type peptide,

the reverse of the proposed experiments for PSA. Though our aim is to identify epitopes that will increase the activation of our T cell hybrid, this result suggested that an increase in the activation of our hybrid would be accompanied by a subsequent increase in β-galactosidase production. This result demonstrates that T cell receptor mutants as well as MHC binding mutants can be identified by the hybrid system. This work is being incorporated in a manuscript in preparation describing the use of hybrids to improve the epitopes of PSA as described below.

The generation and characterization of the mutant library was an important task in our statement of work. In the previous year we had produced the position 1 library. The position 1 (P1) library has been extensively analyzed and over 72 clones sequenced. As expected there are a variety of substitutions using the saturation mutagenesis technique thus validating the approach. In these experiments we made a random series of mutations in the oligonucleotides corresponding to P1 Histidine of HL-10. These were cloned and 72 of the resulting colonies were picked, plasmids isolated, and the sequence corresponding to the epitope was determined. The data previously reported are summarized in the table below.

A.A. Substitution	Theoretical/72	Experimental/72
A	4.5	5
C	2.25	1
D	2.25	2
E	2.25	0
F	2.25	2
G	4.5	13
H	2.25	3
I	3.375	1
K	2.25	0
L	6.75	4
M	1.125	0
N	2.25	0
P	4.5	4
Q	2.25	2
R	6.75	5
S	6.75	9
T	4.5	2
V	4.5	3
W	1.125	0
Y	2.25	4

Figure 2. 72 mutant clones from the position 1 library were picked and the plasmid DNA was sequenced to determine what amino acid substitutions were present in the library.

As can be seen, this strategy very effectively generates mutations and can generate mutations that are even 3 bases different from the wild type. From this analysis, however, it is apparent that some substitutions may be more prevalent than others (e.g. G vs. W). This is likely due to the number of codons coding for the amino acid as well as the GC content of the codon encoding the various amino acids. As expected based on these considerations that not all amino acids are equally represented, it is clear that this mutagenesis approach generates a large variety of random mutations. Moreover, as predicted, no mutations were detected in positions other than

position 1 in this library (data not shown). Thus we have developed a suitable strategy for generating mutations in the target epitope. We have applied this method to the other positions and have successfully generated mutant libraries for the remainder of the positions of HL10, positions 2 through 10. Approximately 10 clones were sequenced from each of these libraries to ensure that the mutagenesis technique had worked as predicted.

Screening of library

After determining that the libraries indeed contained the mutant clones as expected, the clones were individually screened from each library using a 96-well format employing the HL10 reactive T cell hybrid, PSA-HI, identified in Task 1. Clones were scored for activity by counting the number of activated (β-galactosidase expressing) PSA-HI hybrids in the well. The first bar of each chart represents the activity elicited by the wild type construct (Figure 3). More than 80 mutants were screened in each library. Bacterial colonies that resulted in the enhanced stimulation of the hybrid were analyzed further. Plasmids encoding the epitopes were sequenced. The results of the screen as well as the amino acid obtained from the sequence data are presented in Figure 3. Major conclusions from the screen can be summarized.

First, for certain positions, no enhanced mutations could be identified. For example, no amino acids at position 2, 3, 4, 5, 8, or 10 could be identified which improved the recognition of the hybridoma. Second, the screen is robust. Very few false positives (i.e. clones which encode the wild-type amino acid which appear to be enhanced (an example of the rare misidentification is seen in the P3 library). Moreover, the vast majority of the clones identified in the initial screen also retest positive when the peptide is synthesized. Third, multiple independent clones encoding the same amino acid could be identified. For example, in the P1 library several mutants that encoded alanine were identified, but which were encoded by different codons. These data also strongly suggest that it is indeed an enhanced epitope and that the saturation mutageneisis technique worked effectively. Fourth, the degree of enhancement varied dramatically. In some cases we could identify changes which would enhance the recognition modestly (e.g. at position 1 a change from H to S increased recognition 2-3 fold whereas others substitutions the M to N change at position 9 resulted in over a 500 fold improvement in recognition. How this corresponds to MHC binding to T cell recognition was investigated as part of the analysis of the mutants in task 4.

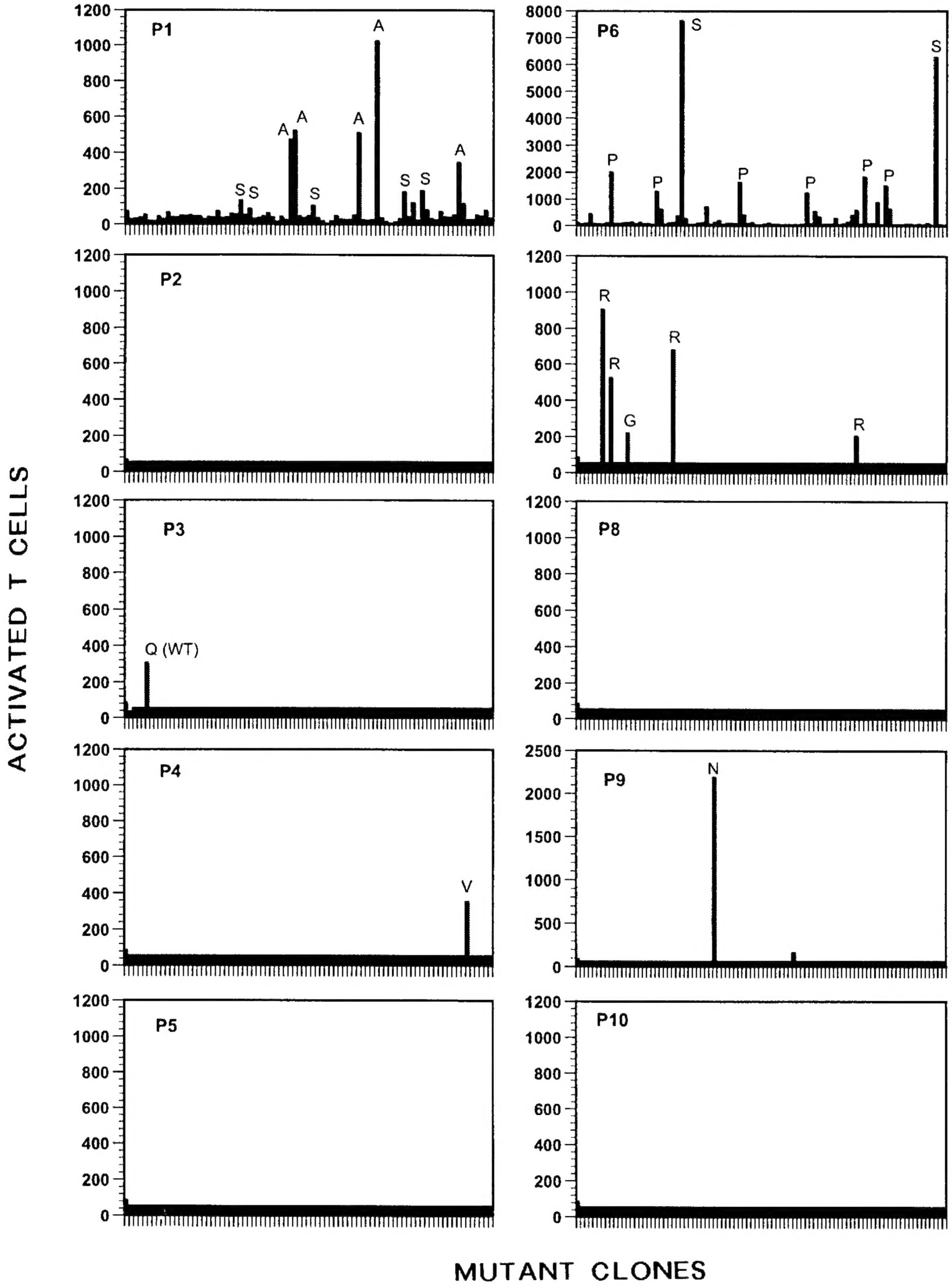

Figure 3: **Library Screen.** HL10 mutant libraries were screened for the ability to stimulate an H-2L^d restricted CTL hybridoma that is specific for wild type HL10, PSA-HI. Recombinant HL10 mutant protein constructs were bound to beads and introduced into cultures containing an H2-L^d expressing antigen presenting cell line, RAW, along with an HL10 specific CTL hybrid, PSA-HI. PSA-HI contains the ß-gal gene under the control of the IL-2 promoter. PSA-HI activation was determined by the number of ß-gal expressing cells/$2x10^5$ PSA-HI, using the hydrolysis of the substrate x-gal to visualize the cells. Wild type HL10 is represented by the first bar in each chart. Note that on this scale the induction by the wild type while positive is low compared to the enhanced mutants.

Analyses using synthetic peptides

Data from the screen strongly suggested that these mutant peptides result in enhanced T cell recognition. However, to obtain more quantitative data and to rigorously examine this issue, we tested these mutants using purified peptides corresponding to the mutation (Figure 4). This allowed us to compare directly the peptides at varying concentrations to determine quantitatively how the altered compared to wild type peptide (HL10). When tested on PSA-HI, all of the mutant peptides were much better than the wild type peptide on a molar basis (see Figure 4).

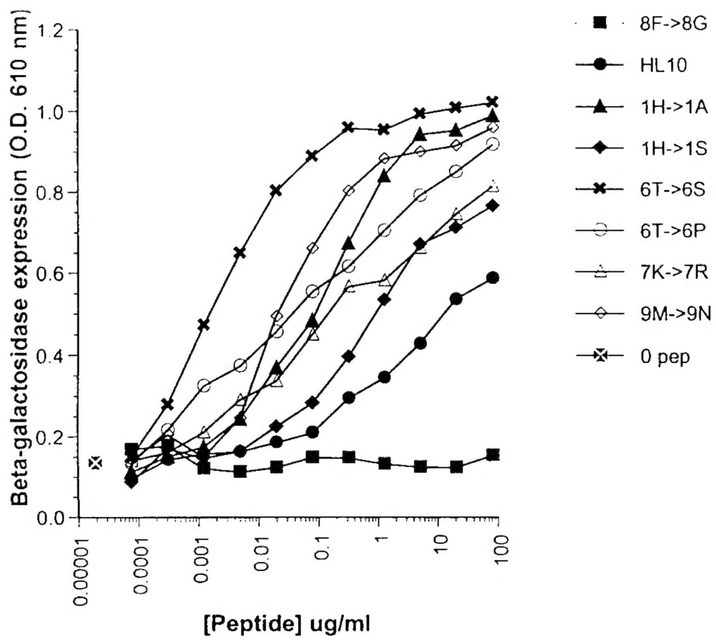

Figure 4: Analyses of APL using purified peptides. Synthetic peptides corresponding to the altered peptide ligands tested in a functional assay. Synthetic peptides corresponding to the APL identified in Figure 3 were tested in a functional assay using the HL10 reactive hybridoma PSA-HI. Decreasing concentrations of peptides were added to 10^5 PSA-HI cells and 10^5 Raw cells as antigen presenting cells as indicated in the figure. Cultures were incubated overnight and beta-galactosidase expression was measured using a colorimetric substrate.

Interestingly, the strength of the synthetic peptides followed the same pattern as seen in the screen. For example, the histidine to serine substitution at position 1 was the least potent of the identified mutants, while the threonine to serine substitution at position 6 was the most potent. Furthermore, the mutants are even more potent than what might be expected from the initial screen. For example serine mutant at position 6 yields an increase in activity of 10,000 fold as compared to the wild type peptide based on the concentration of peptide required to reach the half-maximal activation level of PSA-HI. These results demonstrate that this screen can identify enhanced mutant peptides of a given CTL epitope.

Testing and analysis of peptides: Mechanism of action

Using the screen developed in previous tasks we were able to identify altered peptide ligands with improved ability to stimulate a PSA reactive T cell. Once identified and validated, we evaluated the mechanism by which the peptides were able to enhance the stimulation of the T cell, and determine whether this was due to improved MHC binding or to improved recognition of the T cell receptor. In order to examine the ability of the peptides to bind to MHC molecules, we initially performed a series of experiments looking at the stabilization of the L^d molecule on the mutant cell lines RMAS or T2 that had been transfected with the L^d molecule. These cell

lines lack a functional TAP transporter system necessary for loading peptides onto class I molecules, and as a result, have low levels of class I molecules on the surface. However, these class I molecules can be stabilized by exogenous peptide and the increased stabilization can be measured by flow cytometry. In experiments of this type, it appeared that the AL10 peptide might be a better binder to L^d than HL10. However, this assay was not fully satisfactory as we found no consistent rank order in the ability of the other altered peptides to stabilize L^d, and we could not distinguish the other altered peptide ligands from HL10. In our hands the RMA-S assay is not particularly sensitive, and we hypothesized that these peptides might be rather weak binders. We therefore pursued a functional assay which might be more sensitive than flow cytometry, to examine the ability of the peptides to bind to L^d. For these experiments we developed an MHC binding assay to measure the relative abilities of the peptides to compete for H-2L^d in a competition assay using the H-2L^d binding peptide, $NP_{118-126}$ of LCMV as the indicator peptide (Figure 5).

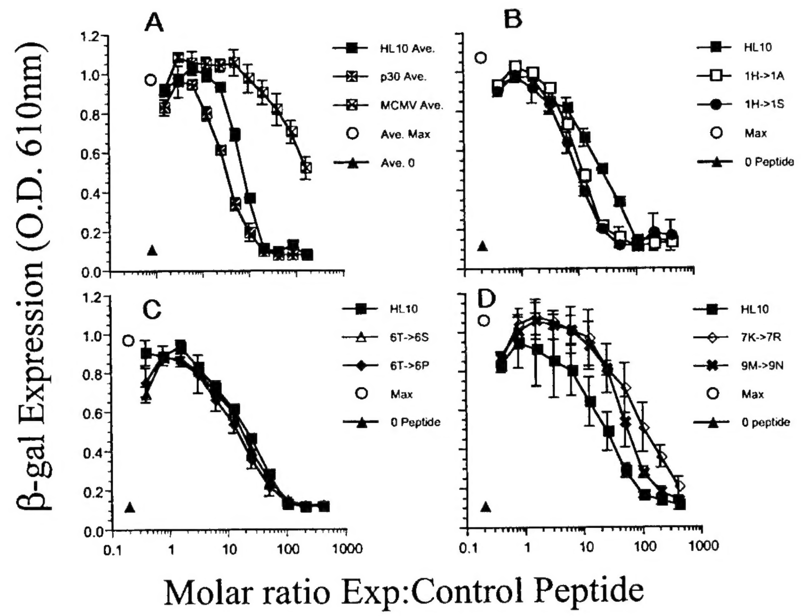

Figure 5: Affinity of the altered peptide mutants for H-2L^d molecules. A competitive binding assay using a NP reactive hybridoma and the $NP_{118-126}$ peptide which binds to Ld was used to determine the relative affinities of the altered peptide ligands. In this assay, the NP hybridoma 1F7 was incubated with a constant amount of NP peptide and the test peptides were added at various concentrations. In the absence of competitor peptide there is maximal stimulation of the hybridoma and as the test peptide competes for binding to L^d, stimulation of hybridoma decreases. Activation of the hybridoma was measured using the beta-galactosidase assay as described in Figure 1.

In this assay, we used an H-2L ^{d} restricted hybridoma specific for the NP $_{118-126}$ LCMV peptide that we developed. Test peptides were added to the hybridoma at various concentrations in the

presence of a constant amount of $NP_{118-126}$, and the degree of activation of the LCMV reactive hybridoma determined. The level of hybrid activation is inversely proportional to the strength of the test peptide binding, since the test peptide is competing for binding with $NP_{118-126}$. Note that in this assay, the T cell hybridoma acts as the H-2L^d expressing antigen presenting cell. We examined the ability of the mutant peptides to compete with the LCMV peptide. These experiments are shown in Figure 5, which for clarity, are separated into four panels. In order to validate the assay, the binding of the wild type HL10 peptide was compared to two previously described L^d binding peptides, APQKAGGFLM, a peptide derived from mouse phosphoglycerate kinase, called p30, which binds with high affinity to L^d, and a MCMV peptide $pMCMV_{168-176}$, which binds weakly to L^d (3). Using this newly developed assay, we found that HL10 binds to H-2L^d approximately 3.5 fold less well than p30 peptide and much better than the $pMCMV_{168-176}$ peptide. By these analyses we found that the 1A and 1S mutants bind H-2L^d molecules with a higher affinity than HL10 (Figure 5b) while mutants 6P and 6S bind to H-2L^d with a similar affinity as HL10 (Figure 5c). Strikingly, two of the mutant peptides (7R or the 9N), bound L^d much less well than HL10 (Figure 5d). These results suggest that HL10 mutants, 7R and 9N, result in a better interaction of the peptide with the T cell receptor of PSA-HI. Thus our screen in TASK 3 identified two classes of altered peptide ligands, one of which acts by binding better to the L^d molecule, and another class of mutants that appear to bind less well, but nevertheless are even more stimulatory on a molar basis because they interact better with the T cell receptor.

Cell mediated responses. We performed a series of experiments to examine the efficacy of the altered peptide ligands to induce cell-mediated responses. In one type of experiment, mice were immunized with the altered peptide ligand, re-stimulated with the same peptide, and analyzed the effector cells generated in CTL assays. We found that many of the effectors generated reacted with targets pulsed with either the altered peptide ligands or with HL10 target, and to a lesser extent, with the H-2^d P815 targets transfected to express PSA. However, the altered peptide ligands did not seem to be superior to the wild type HL10 peptide at generating CTL that reacted with PSA producing targets, and a significant amount of the reactivity appeared to be specific to the altered peptide. We speculated that the re-stimulation *in vitro*, which was required to examine CTL activity, might be biasing the response. Therefore, in order to circumvent this problem, we assessed the ability of the improved epitopes to engender an improved cellular response using the gamma interferon ELI-spot assay without a re-stimulation step. The ELI-spot assay directly determines the frequency of responding cells, and we hypothesized that an improved peptide should increase the frequency of responding cells. Moreover, since the ELI-spot measures cellular responses directly *ex vivo*, it avoids any biases introduced by the *in vitro* amplification step. In light of the hypothesis that the initial priming event is limiting the response, we evaluated priming with the altered peptide, the wild type HL10 peptide, as well as a mixture of the altered peptide ligand mixed with the wild type peptide, to induce cell mediated responses. Dose response experiments determined a low dose of HL10 peptide that was still capable of inducing a consistent response. We used this dose of peptide to examine the ability of the APL to induce cell-mediated responses. A representative example of this experiment is below (Figure 6).

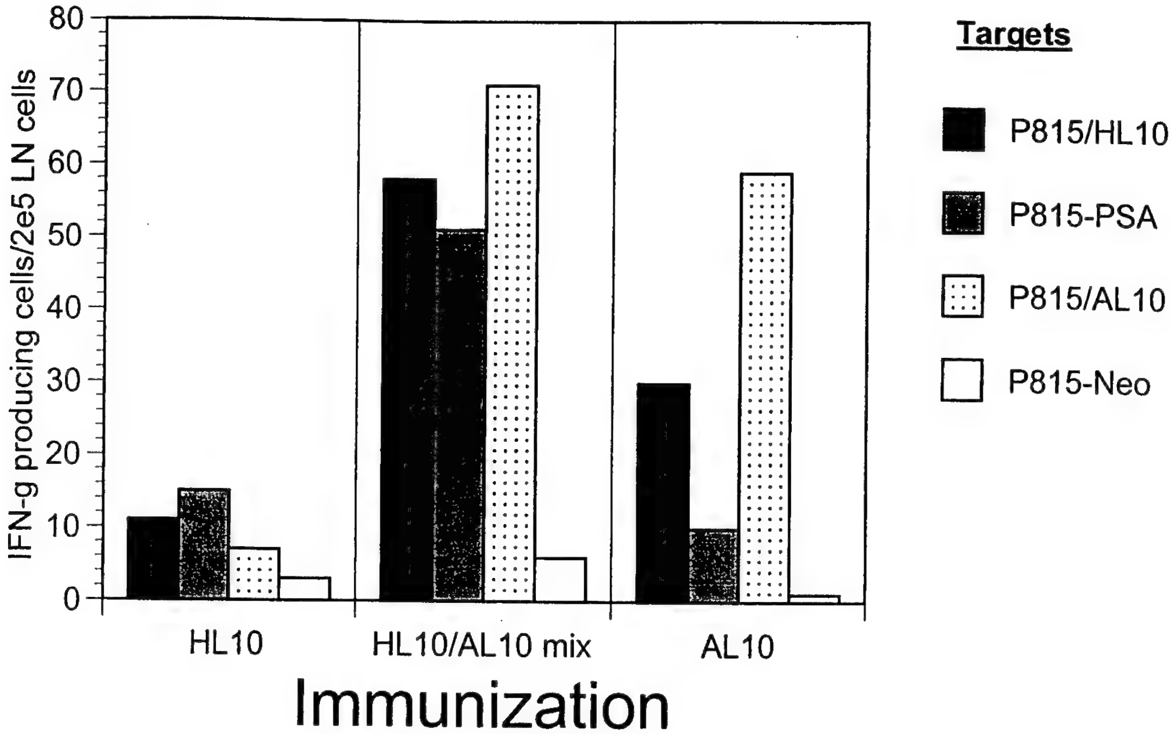

Figure 6: **Immune response to altered peptide ligands.** Immunization with an altered peptide ligand of HL10 enhances the frequency of PSA reactive T cells. Mice were immunized with either 10 micrograms of HL10, or AL10, or a mixture of 5 micrograms of HL10 and AL10. Gamma interferon producing T cells were analyzed directly *ex vivo* from spleen cells seven days after immunization using the gamma interferon ELI-spot technique. The stimulators are as indicated, either P815 cells, P815 cells pulsed with either HL10 or with AL10, or P815 cells which had been transfected to express PSA.

There are several conclusions that can be drawn from these experiments. First, all of these immunizations resulted in specific T cell responses. There is little production of gamma interferon in response to P815 cells which have been transfected with the control vector (designated P815-neo in the Figure), whereas there is substantial gamma interferon production of the effectors with the HL10 pulsed P815 cells. As expected, there is also substantial gamma interferon production observed with the AL10 pulsed target cells in all the immunizations. Interestingly, there is a higher frequency of reactive cells with the AL10 pulsed targets from effectors derived from mice immunized with AL10, compared to mice immunized with HL10. This result suggests that there is a class of T cells that does not cross-react between AL10 and HL10. We hypothesize that it is this class of T cell, one that recognizes AL10, but not HL10, that was preferentially boosted in the re-stimulation experiments. Most strikingly, is that the mixture of HL10 and AL10 results in a much higher frequency of cells reacting with the P815 cells expressing PSA endogenously, than with either peptide alone. This is consistent with the model that the initial event is the triggering of T cells by the AL10 peptide, and those capable of cross-reacting are then expanded by the HL10 peptide. Moreover, these results suggest immunization with a mixture of altered peptide ligand and the wild type peptide would be beneficial when using APL to generate effectors that are capable of responding to tumor cells which express the target antigen of interest.

KEY RESEARCH ACCOMPLISHMENTS

- Characterization of an epitope of PSA
- Development of a methodology for generating mutant libraries
- Characterization of mutant libraries by DNA sequencing
- Demonstration of feasibility of the screening technique
- Functional screen of the 10 mutant libraries
- Identification of altered peptide ligands resulting in increased activation of a PSA reactive hybridoma
- Demonstration and validation of enhanced activation using synthetic peptides
- Analysis of the mechanism of enhanced epitopes
- Demonstration that immunization with mixture of APL and the wild-type peptide increases the number of T cells responding

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations:

Turner, M. J., C. S. Abdul-Alim, R. A. Willis, T. L. Fisher, E. M. Lord, and J. G. Frelinger. 2001. T-cell antigen discovery (T-CAD) assay: a novel technique for identifying T cell epitopes. *J Immunol Methods 256:107*

C. S. Abdul-Alim, M.J. Turner, M. Nocera, M., R. K. Barth, E. M. Lord, and J. G. Frelinger. 2003 Development of a high-throughput method to identify altered peptide ligands with enhanced ability to stimulate T cells (manuscript in preparation).

Posters and Presentations:
Exploiting cross-presentation to characterize T cell epitopes. Turner, M.J., Abdul-Alim, C.S., Lord, E.M. and Frelinger, J.G. Upstate New York Immunology Conference. (Oral presentation)

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Employment or research opportunities applied for and/or received on experiences/training supported by this award: Research training for Ms. Andrea Brooks, a research technician who was supported by this grant. Ms. Brooks is currently applying to graduate school and her research experiences will enhance her chances of being admitted. Advanced research training for Dr. Nocera, a postdoctoral fellow in the laboratory, was provided. She has now taken a position as a senor postdoctoral fellow in a cancer research laboratory. A year-long training experience for an undergraduate was also provided. She is currently enrolled in Case Western Reserve Medical School and plans on a career in research.

Salary Support for Personnel Provided by this Grant
Dr. John Frelinger
Dr. Richard Barth
Dr. Edith Lord
Dr. Mary-Anne Nocera
Ms. Andrea Brooks

Conclusions
The specific conclusions that can be drawn from these experiments are:

1. T-cell hybridomas can be characterized using the solid phase assay developed.

2. The hybridomas have been used to identify a class I restricted peptide of PSA called HL10.

3. An efficient means of generating mutations has been developed and used to develop ten mutant libraries corresponding to each position of a T cell epitope of PSA.

4. Screening of the libraries identified altered peptide ligands with enhanced ability to stimulate a PSA reactive hybridoma.

5. Synthetic peptides have validated the fidelity of the screen.

6. A rapid competitive assay has been developed and employed to assess MHC binding.

7. Analysis of the altered peptide ligands revealed both improved MHC binders as well as one with improved interactions with the T cell receptor.

8. Immunization analyses showed that a mixture of an altered peptide ligand and the wild-type peptide increases the frequency of reactive T cells revealed by ELI-spot analyses.

Taken together, these findings support the hypothesis that altered peptide ligands can be discovered using the novel methodology developed. By screening libraries we have constructed, and synthesizing the corresponding peptides, we have identified several altered peptide ligands with an increased ability to stimulate a PSA reactive T cell hybridoma. Moreover, using these peptides in immunization strategies can result in an increased T cell response. The studies have firmly established "proof of principle" that this method can be used to improve T cell epitopes of PSA. This methodology should be applicable to other tumor antigens.

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2. Jameson, S. C., and M. J. Bevan. 1992. Dissection of major histocompatibility complex (MHC) and T cell receptor contact residues in a Kb-restricted ovalbumin peptide and an assessment of the predictive power of MHC-binding motifs. *Eur J Immunol 22:2663.*
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Recombinant Technology

T-cell antigen discovery (T-CAD) assay: a novel technique for identifying T cell epitopes

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Abstract

The identification of T cell epitopes is a critical step in evaluating and monitoring T cell mediated immune responses. Here, we describe a novel technique for simultaneously identifying class I and class II MHC restricted epitopes using a one-step protein purification system. This method uses Ni/chelate coated magnetic beads and magnetic separation to isolate poly-histidine tagged recombinant antigen from bacterial lysates. These beads, once coated with antigen, are also used to deliver antigen to APC where it is processed and presented to T cells. A colorimetric assay and ovalbumin specific, lacZ inducible, T cell hybridomas were used to validate the system. Further, using PSA specific hybrids, generated from T cells isolated from PSA secreting tumors, both class I and class II MHC restricted epitopes of PSA were identified. Additional characterization has shown that these peptides contribute significantly to the overall PSA specific response in vivo, and may represent the dominant epitopes of PSA. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Prostate-specific antigen; T cell epitopes; Major histocompatability complex

1. Introduction

The immune response is characterized by the response to small portions of molecules called epitopes. For T cells, these consist of short peptides bound to molecules of the major histocompatibility complex (MHC). In the case of cytotoxic CD8 + T cells, these peptides are generally 8–10 amino acids in length and are complexed with class I MHC molecules (Falk et al., 1991; Rammensee, 1995). Similarly, CD4 + or helper T cells generally recognize peptides of 12–25 amino acids in length complexed with class II MHC molecules (Rudensky et al., 1991; Rammensee, 1995). The characterization of such epitopes has led to an enormous increase in the understanding of T cell recognition and activation. Knowledge of these peptides has also greatly aided the in vivo analysis of T cell responses and may also have therapeutic utility. For example, newly developed tetramer technology, in which soluble la-

Abbreviations: APC, antigen presenting cells; PSA, prostate-specific antigen; NF-AT, nuclear factor activating T cells; MHC, major histocompatibility complex; T-CAD assay, T-cell antigen discovery assay; DHFR, dihydrofolate reductase; Ova, ovalbumin

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beled complexes of MHC molecules are made using a single peptide, has made it possible to analyze the dynamics of T cell responses to HIV and other viruses using flow cytometry (Altman et al., 1996; Murali-Krishna et al., 1998; He et al., 1999). Further, peptide based vaccines are being developed for clinical use in the treatment of HIV and for cancer therapy (Porgador and Gilboa, 1995; Porgador et al., 1996; Brossart et al., 1998; Kundu et al., 1998; Morse et al., 1998; Nair et al., 1999). In addition, vaccinations using peptides which act as antagonists are under consideration for the treatment of autoimmune diseases (Sette et al., 1994; Brocke et al., 1996; Nicholson et al., 1997; Bielekova et al., 2000; Kappos et al., 2000). All of these potential uses contribute to the need for identifying epitopes at the molecular level.

Several strategies have been successfully employed for identifying T cell epitopes. These include peptide elution from MHC molecules, the synthesis of peptides based on motif predicting computer algorithms for particular MHC alleles, DNA transfer, as well as the use of recombinant viruses as expression vectors (De Plaen et al., 1988; Falk et al., 1991; Jardetzky et al., 1991; Rudensky et al., 1991; Hunt et al., 1992; Henderson et al., 1993; Castelli et al., 1995; Valmori et al., 2000). While significant advances have been made using these approaches, they are often technically demanding, dependant on specialized equipment, and specific for a given class I or class II molecule. Therefore, a generalized method that could more easily characterize both class I or class II restricted epitopes would be extremely valuable for fundamental immunology studies as well as for the development and analysis of vaccines.

We set out to develop a method based on recent advances in understanding antigen presentation mechanisms. It is generally accepted that exogenous antigens originating from outside of antigen presenting cells are preferentially directed towards the class II pathway, whereas antigens derived from within the cell are directed to the class I pathway (Brodsky and Guagliardi, 1991; Monaco, 1995). However, it has been known for many years that there are exceptions to this generalization in which exogenously derived antigen can be presented in the context of class I molecules (Bevan, 1976; Gooding and Edwards, 1980; Yewdell and Bennink, 1999). Recently, this phenomenon, called cross-priming or cross-presentation, has received additional attention because of its role in the generation of anti-tumor responses and in peripheral tolerance (Huang et al., 1994; Pulaski et al., 1996; Kurts et al., 1997; Reimann and Schirmbeck, 1999). In studying this process, our laboratories, as well as others (Kovacsovics-Bankowski et al., 1993; Reis e Sousa and Germain, 1995; Storozynsky et al., 1999) have found that particulate antigens, in the form of beads, are very effective in delivering exogenous antigen to the class I pathway. Further work demonstrated CD4+ T cell hybridomas could also be activated by antigen presenting cells (APC) that have taken up antigen coated beads (Shen et al., 1997; Storozynsky et al., 1999). Thus, beads appear to be a very efficient way of delivering antigen for presentation to both class I and class II restricted T cells.

In the current study, we have taken advantage of these findings to develop a novel assay for identifying T cell epitopes. By using 6 × His tags to attach recombinant proteins to magnetic beads that are also used for antigen isolation, we have developed an easy way of delivering antigens to APC for processing and presentation. In the current study, we have used this approach in conjunction with a colorimetric assay for T cell activation to illustrate the feasibility and validity of this one-step approach. We have termed this technique the T cell antigen discovery (T-CAD) assay, and have used it to identify both class I and class II epitopes of human prostate-specific antigen (PSA).

2. Materials and methods

2.1. Mice and cell lines

BALB/cByJ (H-2^d), C57BL/6J (H-2^b), and [BALB/cByJ × C57BL/6J] F_1 (H-2^{dxb}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and used at 8–10 weeks of age. Ovalbumin (Ova) specific T cell hybridomas B3Z86/90.14 (H-2 K^b) (Shastri and Gonzalez, 1993) and BDZ.21.2 (H-2 I-A^d) (Storozynsky et al., 1999), and PSA specific T cell hybridomas PSA-HI and PSA-HII were grown as described previously (Storozynsky et al., 1999). Line 1/PSA/IL-2 (H-2^d) and B16/PSA (H-2^b) cell lines were generated by transfecting the

cell lines Line 1/IL-2 (McAdam et al., 1995) and B16.F0 (ATCC CRL 6322) with the mammalian expression vector pHβ-PSA (Wei et al., 1996). PSA transfectants, Line 1/PSA (H-2^d) and P815/PSA (H-2^d), were characterized previously (Wei et al., 1996). L cells expressing the L^d molecule were provided by Dr. Jeffrey Frelinger (University of North Carolina, Chapel Hill). The B cell lymphoma cell line M12 and the M12 derived class II mutants, M12.A2 (I-A^{d+}) and M12.B5 (I-E^d +), were developed by Dr. Laurie Glimcher and supplied to us by Dr. Alexandra Livingstone (University of Rochester) (Glimcher et al., 1985).

2.2. Fusion protein constructs and peptides

Recombinant proteins were generated using the bacterial expression vector pQE-40 (Qiagen, Valencia, CA, USA), which contains a 6 × His coding region as well as the murine dihydrofolate reductase gene (DHFR). The pQE-40 Ova-DHFR vector (Ova/DHFR) was constructed by subcloning the coding region for amino acids 256–359 of Ovalbumin (Ova) from the pEVRFO-Ova plasmid using polymerase chain reaction (PCR) and specific primers (Shastri and Gonzalez, 1993). The insert was subcloned into the pQE-40 vector using the Sal I and Kpn I restriction sites designed into the primers. Human PreProPSA (PSA) was produced by PCR using the pHβ-PSA expression vector and PSA specific primers that contain Bam HI and Hind III restriction sites in the 5′ and 3′ primers, respectively. The insert was subcloned into the pQE-40 vector, resulting in the removal of the DHFR coding region. This subsequently creates a 6 × His/PSA fusion protein. PSA deletion constructs were generated in a similar fashion by amplifying 3′ deletions of PSA using a constant 5′ primer and five individual 3′ primers starting at 736 base pairs (bp), 610 bp, 505 bp, and 394 bp. Synthetic peptides PSA 238–253 (ERPSLYTKVVHYRKWI), PSA 188–197 (HPQKVTKFML), Ova 257–264 (SIINFEKL), and Ova 323–339 (ISQAVHAAHAEINEAGR) were synthesized by Macro-Molecular Resources (University of Colorado, Fort Collins, CO, USA).

2.3. Protein production

Production of recombinant DHFR, Ova-DHFR and PSA deletion construct fusion proteins were performed as described by the manufacturer (Qiagen). Recombinant proteins were isolated by lysing the bacterial pellets with 8 M urea (pH 7.5) and 0.5 ml of bacterial lysates were used for conjugation to Ni/chelate paramagnetic beads (Bangs Laboratories, Fishers, IN, USA). To load the magnetic beads, 2×10^9 Ni/chelate beads were added to 0.5 ml of bacterial lysates containing Ova/DHFR, DHFR, or PSA deletion constructs independently for 1 h. The adsorbed beads were isolated by magnetic separation, washed twice and diluted to 2×10^7 beads/μl. PSA deletion construct production was confirmed by Western blot analysis using a rabbit anti-PSA polyclonal antibody (DAKO, Carpinteria, CA, USA) and standard immunoblot techniques (Baecher-Allan et al., 1993).

2.4. Generation of T cell hybridomas

Ova specific T cell hybrids B3Z86/90.14 (B3Z) and BDZ.21.2 (BDZ) were generated as described previously (Shastri and Gonzalez, 1993; Storozynsky et al., 1999). PSA specific class I restricted T cell hybridomas were generated by fusing Thy-1 + tumor infiltrating lymphocytes (TIL) isolated from BALB/c mice challenged with Line 1/PSA/IL-2 tumors to the T cell fusion partner BWZ.36 as described (Sanderson and Shastri, 1994a; McAdam et al., 1995). Class II restricted T cell hybridomas were generated using a similar protocol. For the class II hybridomas, Thy-1 positive T cells for making T cell hybrids were generated in [BALB/cByJ × C57BL/6J] F_1 mice immunized twice with 1×10^7 irradiated (5000 rad) B16/PSA cells and challenged with Line 1 PSA tumors. Tumor infiltrating lymphocytes were isolated and fused as above.

2.5. Antigen presentation assays

Antigen presentation assays were performed by culturing 2×10^5 T cell hybrids with 1×10^5 APC. APC used were [BALB/cByJ × C57BL/6J]F_1 (H-$2^{d \times b}$) bone marrow derived dendritic cells (Inaba et al., 1992), RAW 264.7 cells (H-2^d, class I + and class II +) activated with 100 units/ml of IFN-γ (R + D Systems, Minneapolis, MN, USA), or MHC mutant cell lines as described in the figure legends. Recombinant antigens used were Ni/chelate beads conjugated with Ova/DHFR, DHFR, or the individual PSA deletion constructs isolated from bacterial

lysates as described above and used at 6×10^7 beads/well. Experiments were also performed in the presence of 3 μM synthetic peptides Ova 257–264, Ova 323–339, PSA 188–197, PSA 238–253 or 3 μM whole Ova (Sigma, St. Louis, MO, USA) or PSA protein (Cortex, San Leandro, CA, USA). After 16–18 h cultures were developed using the β-galactosidase substrate X-gal (Fisher Biotech, Pittsburgh, PA, USA) and the number of activated hybrids was quantified (Storozynsky et al., 1999). Assays using the class I restricted hybrids and particulate antigen were performed in the presence of 100 μM chloroquine (Sigma) to enhance cross presentation (unpublished results).

2.6. Functional assays

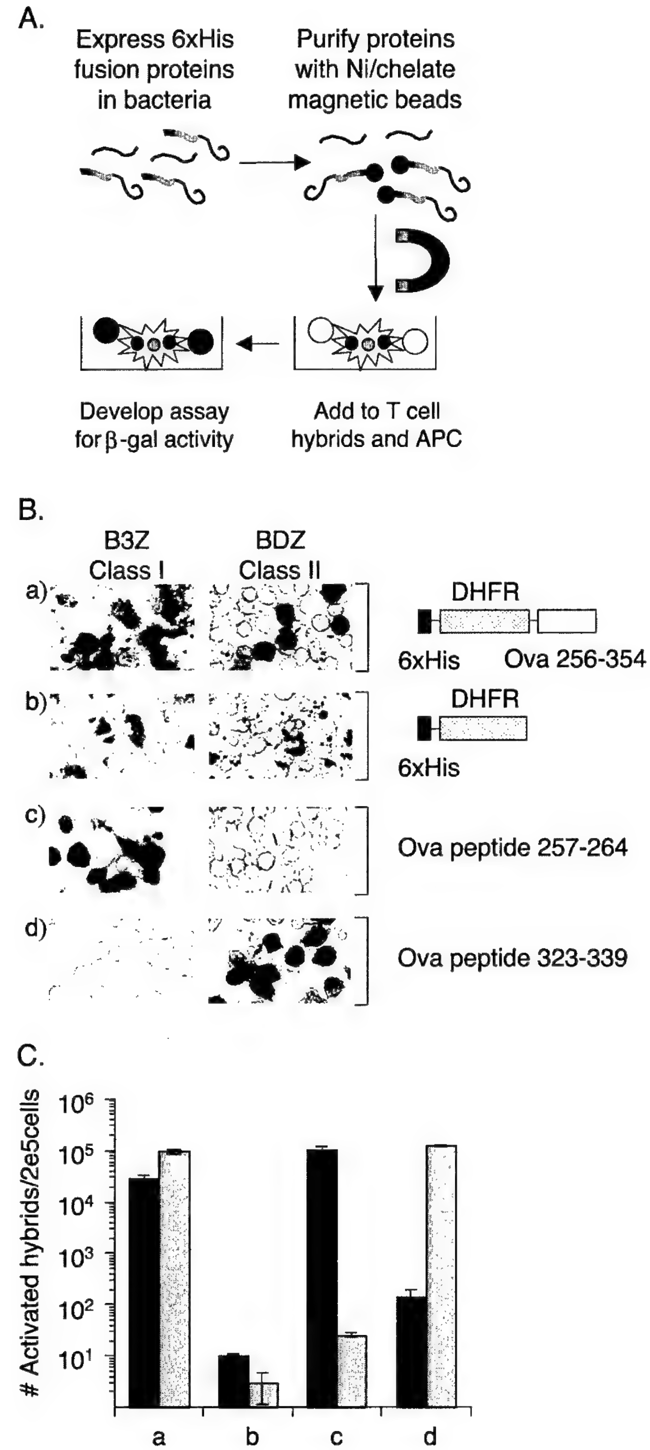
BALB/cByJ mice were injected with 2×10^5 Line 1/PSA/IL-2. After 15 days, the TIL were isolated and evaluated for their ability to lyse targets expressing full length PSA protein (P815/PSA), P815 transfected with an irrelevant antigen (P815/neo), or P815/neo pulsed with 50 μg/ml of PSA peptide 188–197. Cytotoxicity assays were performed as previously described (McAdam et al., 1995). Proliferation assays were performed by immunizing BALB/cByJ mice in the footpad with 80 μg of recombinant PSA emulsified in complete Freund's adjuvant. Ten days later, the popliteal lymph nodes were harvested and 5×10^5 lymph node cells/well were cultured with various dilutions of PSA protein (Cortex) or PSA peptide 238–256 starting at 0.83 μM. After 72 h, the cultures were pulsed with 1 μCi of ^{3}H-Thymidine for 16 h and analyzed for thymidine incorporation. All animal studies were performed in compliance with the University of Rochester Committee on Animal Resources.

3. Results and discussion

3.1. Development and validation of the T cell antigen discovery (T-CAD) assay

We set out to develop a system that could be used to identify both class I and class II restricted T cell epitopes. In designing this system, several considerations were taken into account. First, since T cells recognize short peptides complexed with MHC molecules, and thus are not dependent on the native conformation of the protein, we reasoned that bacterially expressed recombinant proteins could be used as a source of antigen. Therefore, recombinant proteins were engineered to contain N-terminal $6 \times$ His tags so that the antigen could be easily purified from bacterial lysates. The $6 \times$ His tag allows the antigen to be adsorbed onto small nickel coated paramagnetic beads and purified from endogenous bacterial proteins by magnetic separation even under strong denaturing conditions necessary for isolating antigen from inclusion bodies. An additional advantage of using beads is that particulate antigen is remarkably efficient at targeting antigen for presentation to T cells (Shen et al., 1997; Storozynsky et al., 1999). Combining these features, the antigen-coated beads were used for antigen presentation in a simple one-step procedure (Fig. 1A). As a readout of T cell activation, T cell hybridomas were used that can be analyzed enzymatically by colorimetric substrates. These hybrids contain the *Escherichia coli* β-

Fig. 1. Development and validation of the T-CAD assay. (A) Illustration of the T-CAD assay. $6 \times$ His tagged recombinant proteins are purified directly from bacterial lysates using Ni/chelate coated paramagnetic beads. The protein-coated beads are "fed" to APC in culture with T cell hybridomas, allowing processing and presentation of the proteins. After 16–18 h, activated T cell hybrids are detected using the X-gal substrate, which results in an insoluble blue precipitate within the activated hybridomas. (B) Activation of Ova specific class I and class II restricted T cell hybridomas using the T-CAD assay. T-CAD assays were performed as described in Section 2.5. Cultures in the left-hand column used the B3Z class I restricted hybridoma and cultures in the right-hand column used the class II restricted hybridoma BDZ. All cultures used bone marrow derived dendritic cells from [BALB/cByJ $\times$ C57BL/6J]F_1 mice as APC, and activity was detected using the X-gal substrate. The antigens are as indicated: (a) $6 \times$ His-Ova/DHFR fusion protein adsorbed to Ni/chelate magnetic beads; (b) $6 \times$ His/DHFR fusion protein adsorbed to Ni/chelate magnetic beads; (c) 3 μM Ova class I peptide, Ova 257–264 peptide that activates with B3Z; (d) 3 μM Ova class II peptide, Ova 323–336 peptide that activates BDZ. (C) The same cultures shown above in (B) were examined, and the number of activated cells was determined. Black bars indicate the number of activated cells for the B3Z class I hybridoma and the gray bars represent the activated cells using the BDZ class II hybridoma. The antigens (a–d) are the same as in (B).


A.
Express 6xHis fusion proteins in bacteria
Purify proteins with Ni/chelate magnetic beads
Develop assay for β-gal activity
Add to T cell hybrids and APC
B.
B3Z Class I
BDZ Class II
a)
b)
c)
d)
DHFR
6xHis
Ova 256-354
DHFR
6xHis
Ova peptide 257-264
Ova peptide 323-339
C.
Activated hybrids/2e5cells
10^6
10^5
10^4
10^3
10^2
10^1
a
b
c
d

galactosidase gene under the control of the IL-2 regulatory element NF-AT. Normally, activated T cells are rapidly induced to synthesize IL-2, a process that involves transcriptional activation mediated by factors binding the NF-AT regulatory region. This also occurs in the hybrid system, and due to the reporter gene fusion, the activated hybrids will also synthesize β-galactosidase (Karttunen and Shastri, 1991; Sanderson and Shastri, 1994b; Storozynsky et al., 1999). This allows T cell activation to be easily assessed using chromogenic β-galactosidase substrates such X-gal (Fig. 1A).

Initial experiments were performed using the well-characterized Ova specific T cell hybridomas B3Z and BDZ that recognize the Ova 257–264/K^b and Ova 323–339/I-A^d complexes, respectively (Sanderson and Shastri, 1994b; Storozynsky et al., 1999). To evaluate the ability of the Ova specific hybrids to perform in the T-CAD assay, recombinant Ova fusion proteins were synthesized using the bacterial expression vector pQE-40. The Ova/DHFR construct encodes an N-terminal $6 \times$ His tag, the murine dihydrofolate reductase protein (DHFR), and a region of Ova (256–354) that contains both of the reported class I and class II restricted T cell epitopes. As shown in Fig. 1B (panel a) the Ova specific T cell hybrids are specifically activated by the Ova/DHFR protein purified directly from bacterial lystes using the Ni/chelate magnetic beads. The specificity of this response is illustrated by the lack of activation when the hybrids are cultured with magnetic beads, which appear as reddish brown aggregates, that are adsorbed with the DHFR construct alone (Fig. 1B, panel b). The numbers of activated cells in Fig. 1B were also quantified, and the results are shown in Fig. 1C. There was a dramatic increase in the number of activated hybrids when the cells were cultured with the Ova containing fusion protein compared with the DHFR protein alone. These results show that recombinant proteins can be isolated from bacterial cultures and effectively presented to T cell hybrids by APC, validating the T-CAD system.

3.2. Generation and characterization of PSA reactive hybrids

The results obtained above with the well characterized model antigen Ova show that bacterially expressed recombinant proteins can be presented by professional APC to both class I and class II restricted T cell hybridomas, leading to the hypothesis that this system could be used to identify unknown T cell epitopes. Because of our interest in prostate cancer immunotherapy, we set out to characterize the immune response to tumors expressing human prostate-specific antigen (PSA) as a model tumor antigen. To characterize the PSA specific immune response, a panel of PSA specific hybridomas was generated by fusing the BWZ.36 (lacZ+) T cell fusion partner with PSA specific T cells derived from mice immunized with PSA secreting tumors (see Section 2.4). Two hybridomas generated from these fusions, PSA-HI and PSA-HII, were selected for more detailed analysis. The T cell hybridoma, PSA-HI, was specifically activated by the tumor cell line P815 (H-2^d), transfected to express human PSA, but not the parental P815 tumor transfected with the expression vector alone (Table 1). Additionally, the hybrid was not activated by presentation of soluble PSA or Ova by APC (Raw 264.7 cells), but was activated by APC that were presenting the PSA derived from PSA coated beads. These data strongly

Table 1
Characterization of PSA specific T cell hybridomas PSA-HI and PSA-HII

Hybrids	Antigen					
	P815 PSA	P815 neo	Soluble PSA	Soluble Ova	PSA beads	Ova/DHFR beads
PSA-HI	+ + +	–	–	–	+ +	–
PSA-HII	–	–	+ + + +	–	+ + + +	–

Assays were performed as described in Section 2.5. About 2×10^5 T cell hybrids were cultured with 1×10^5 P815 cells expressing either PSA or vector control (neo), or RAW 264.7 cells pulsed with 3 μM PSA/Ova or 6×10^7 beads/well. + indicates cultures containing activated hybridomas, with each + indicating approximately a tenfold increase in positive cells.

suggest that the hybridoma is class I restricted and confirms the ability of these hybrids to be activated by cross-presentation. A second hybridoma, PSA-HII, reacts specifically with soluble PSA after processing by APC, or with APC that have taken up PSA adsorbed Ni/chelate beads (Table 1). However, it does not react with other antigens such as Ova or with the class II negative P815 cells transfected with PSA, suggesting that the hybridoma is both class II MHC restricted and PSA specific.

3.3. Generation of PSA deletion constructs and analysis of PSA using the T-CAD assay

To identify the regions of PSA recognized by the T cell hybrids, a full-length cDNA construct was constructed encoding PSA as well as a series of deletion constructs in the pQE-40 bacterial expression vector. These constructs contain the $6 \times$ His region described above, followed by PSA peptides of varying length and including the signal peptide, pro-

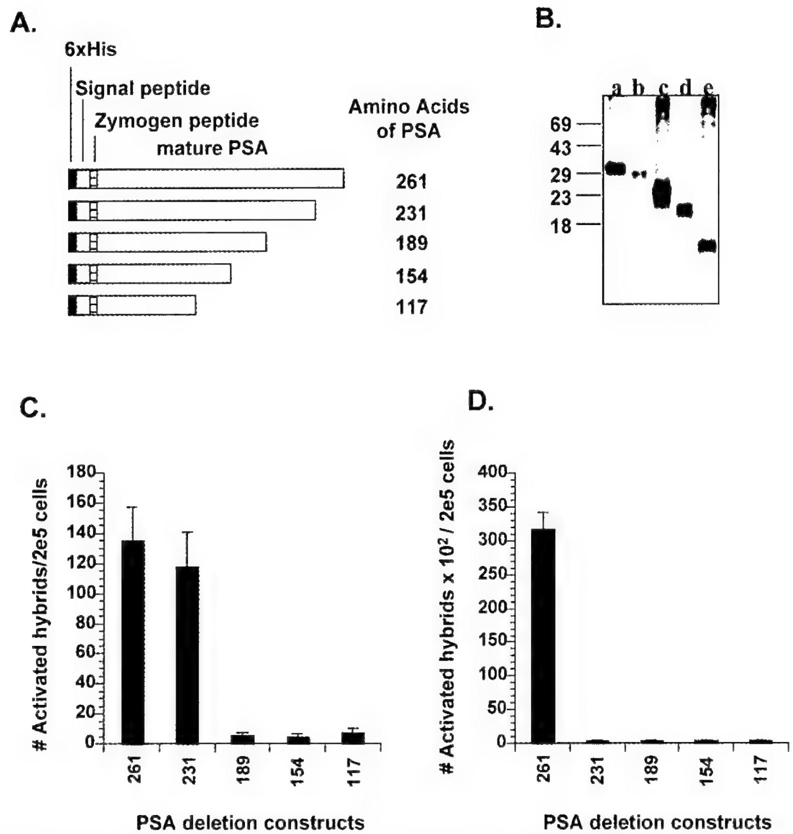


Fig. 2. Generation of PSA deletion constructs for T cell epitope identification. (A) Schematic of the PSA constructs used in the PQE40 bacterial expression vector. The black box indicates the $6 \times$ His Tag, the gray box indicates the signal peptide, the hatched box indicates the zymogen or pro peptide, and mature PSA is designated by the open box. (B) Immunoblot analysis of the PSA deletion constructs. Recombinant PSA deletion constructs were synthesized and detected using a rabbit anti-human PSA antiserum as described in Section 2.3. Molecular weight markers are indicated and represent apparent molecular weight in thousands. The recombinant proteins used were designated by the number of amino acids of PSA they encode, starting with the first amino acid designated as number 1 (a) 261, (b) 231, (c) 189, (d) 154 and (e) 117 amino acids (full length). (C) Analysis of the class I restricted PSA specific T cell hybridoma PSA-HI with proteins encoded by the PSA deletion constructs. PSA-HI cells were incubated with RAW 264.7 cells and Ni/chelate beads adsorbed with the various PSA deletion peptides. After incubation, the number of activated cells was determined using X-gal. The numbers on the abscissa indicate the length of the PSA peptides in amino acids adsorbed to the Ni/chelate beads. (D) Analysis of the class II PSA specific hybridoma PSA-HII with proteins encoded by the PSA deletion constructs. Assays were performed as in (C) using the same set of PSA constructs.

peptide, as well as defined C terminal deletions illustrated in Fig. 2A. Bacterial cultures induced for expression of the individual deletion constructs were characterized by immunoblot analysis using a rabbit anti-PSA polyclonal antibody (Fig. 2B). These results showed that the constructs encode proteins of the predicted molecular weight, and they contain epitopes that can be detected with the rabbit anti-PSA polyclonal antibody to human PSA as expected.

Using the T cell hybrids and the rPSA deletion constructs, experiments were performed to identify the class I and class II T cell epitopes of PSA. The class I (PSA-HI) and class II (PSA-HII) PSA specific hybrids were tested against this panel of PSA deletion constructs each conjugated to Ni/chelate beads individually, and presented by the macrophage cell line RAW 264.7 (H-2^d). Antigen presentation assays were performed as described in Section 2, and activated hybrids (blue cells) were scored after staining with X-gal substrate. The results of these assays are depicted in Fig. 2. The class I restricted hybridoma, PSA-HI, is activated by the full length PSA construct and the first deletion construct, but not by the second deletion construct (1–189) indicating that the epitope is contained within the region 189–231 (Fig. 2C). In an analogous fashion, the class II hybrid reacts with the full-length construct, 1–261, but not the first deletion construct, 1–231 (Fig. 2D). This indicates that the epitope is contained within the final 30 amino acids of the carboxy-terminal end of PSA.

3.4. Determination of class I and class II epitopes

Based on the analysis above, the identified region of PSA and an additional 10 amino acids upstream were examined, for potential H-2^d class I MHC binding epitopes. The addition of 10 amino acids included any potential epitopes that might be destroyed if the breakpoint of the deletion occurred within the epitope. By comparison to previously reported H-2^d class I binding epitopes (Corr et al., 1992, 1993; Romero et al., 1994) as well as computer algorithms (Parker et al., 1994), a number of candidate peptides were identified even in this limited region. Three peptides were synthesized and tested in functional assays. One of these peptides PSA 188–197, which was predicted to bind L^d (Rammensee et al., 1995), stimulates PSA-HI in the presence of APCs whereas the others do not (data not shown). In order to determine the restriction element of the peptide definitively, we used L cells, an H-2^k fibroblast cell line, transfected with the L^d gene or vector alone. The results from one such assay are shown in Fig. 3, in which L cells trans-

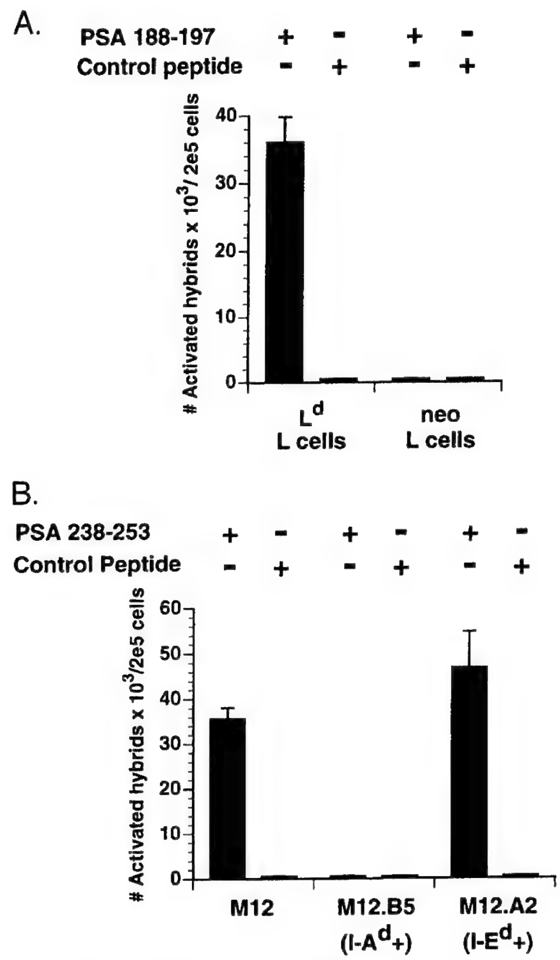


Fig. 3. MHC restriction of PSA peptide epitopes 188–197 and 238–253. (A) Analysis of the class I restricted peptide 188–197. T cell hybridoma PSA-HI was incubated with L cells transfected to express either the MHC class I molecule L^d or the vector alone and pulsed with PSA peptide 188–197 or control peptide. Activated hybridomas were identified by staining with X-gal. (B) Analysis of the class II restricted PSA peptide 238–253. The T cell hybridoma PSA-HII was incubated with the M12 cell line or M12 derived class II mutants M12.B5 (I-A^d +) or M12.A2 (I-E^d +) in the presence of PSA peptide 238–253 or control peptide. Activated cells were identified by staining with X-gal substrate.

fected with the L^d gene were pulsed with the peptide PSA 188–197. The L cells transfected with the L^d class I gene and pulsed with PSA 188–197 were recognized, whereas neither L^d expressing L cells pulsed with irrelevant peptide nor parental L cells, which do not express the L^d molecule, stimulated PSA-HI activation. These results demonstrate that the T cell hybrid PSA-HI is restricted by L^d and is specifically activated by the PSA peptide 188–197. It also illustrates that even with limited information about a particular MHC motif, peptides can be easily identified using this approach.

We performed a similar analysis to the one described for class I recognition to determine the class II peptide recognized by the PSA reactive hybridoma PSA-HII. Although some analysis has been performed to identify class II MHC motifs by crystal structure and mutagenesis (Wall et al., 1994; Fremont et al., 1996), due to the ambiguity of the length of the class II binding peptides, it has been much more difficult to map the precise anchor residues and establish peptide motifs. Therefore, using the same deletion strategy as we have used above, we constructed a series of finer deletion constructs within the region of 174–231, and mapped the class II hybrid to the region 238–253 (data not shown). On this basis, we synthesized the peptide PSA 238–253 and evaluated it in presentation assays analogous to those described above. The PSA-HII hybrid was cultured with the B cell lymphoma cell line, M12, that expresses both I-A^d and I-E^d, or mutant cell lines derived from M12 that express only I-A^d (M12.B5) or I-E^d (M12.A2). As shown in Fig. 3, the hybrid PSA-HII is strongly activated by the parental line that expresses both class II MHC molecules when pulsed with the peptide PSA 238–253, but not an irrelevant peptide. Activation is also seen when the hybrids are cultured with the PSA peptide 238–253 and the mutant cell line M12.A2, which expresses I-E^d but lacks I-A^d. However, little activation is seen using the peptide in conjunction with the cell line that expresses I-A^d and the same PSA peptide. These results demonstrated that the PSA-HII hybridoma is specific for PSA peptide 238–253 restricted by the class II MHC I-E^d molecule. Interestingly, consistent with this analysis, the peptide identified contains several basic residues, which are found in other I-E^d restricted epitopes and may contribute to binding class II MHC (Sette et al., 1989).

3.5. Demonstration that the epitopes identified participate in the in vivo PSA response

The identification of the peptides above used hybridomas made from T cells that arose in animals bearing PSA expressing tumors. This data alone would suggest that these epitopes participate in the PSA specific anti-tumor response. However, to demonstrate this definitively, we analyzed both the CTL and helper T cell responses from animals that had been immunized with PSA. To analyze the CTL response to PSA, CD8 positive T cells were isolated from tumors co-expressing PSA and IL-2, and were assayed for their lytic ability of target cells expressing full length PSA or targets that have been pulsed with the PSA peptide 188–197 alone. IL-2 was included in the tumor challenge to enhance the lytic population of TIL as we have shown previously (McAdam et al., 1995). As shown in Fig. 4, these cells specifically lyse P815 cells expressing PSA targets that have been sensitized with the PSA peptide 188–197, but not the vector control (P815 neo). Additionally, a large part of the PSA specific response can be accounted for using this peptide, which suggests that this may be the dominant class I restricted epitope of PSA in BALB/cByJ mice. To analyze the CD4 positive T cell proliferative response, BALB/cByJ mice were immunized with PSA emulsified in complete Freund's adjuvant and the lymph node cells were isolated and analyzed by proliferation assay for their response to PSA and the PSA peptide 238–253. The results of the proliferation assays are illustrated in Fig. 4, and show that the cells proliferate in response to PSA as well as the peptide PSA 238–253. Additionally, a significant portion of the helper response was directed at this epitope, suggesting that it is an important and perhaps even the dominant class II restricted epitope in the PSA specific response.

The T-CAD assay has a number of features that make it flexible and robust. Unlike most other methods, this method can be used to identify either class I and class II restricted epitopes as we have demonstrated for both Ova and PSA. Further, since the

antigens not only for human pathogens and cancers, but also to autoimmune diseases such as diabetes or multiple sclerosis. In summary, the adaptability of the T-CAD assay to other experimental systems not only for epitope mapping but also antigen identification makes the T-CAD assay a flexible tool for analyzing T cell mediated responses.

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